Bovine Cerebral Theileriosis: First Molecular Confirmed Report in Cross Bred Cattle Calf in India

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Abstract

Bovine tropical theileriosis caused by *Theileria annulata*, is a serious constraint to Indian dairy industry with more fatal infections in exotic cattle and substantial losses to cross-bred and indigenous zebu cattle. The present communication is to place on record the first report of molecular based confirmed case of cerebral theileriosis caused by *T. annulata* coupled with its morphological detection, clinical manifestations, haematological alterations and therapeutic management in a cross bred cattle calf from India. After preparation of peripheral thin blood smear from cross bred cattle calf at the site of collection and fixation with methanol, blood sample brought to Department of Veterinary Parasitology, College of Veterinary Science and A.H, Jabalpur and stained by standard protocol for Giemsa staining. Genomic DNA was isolated from the collected blood sample using QIAamp® DNA blood mini kit following the manufacturer's recommendations and PCR was performed. The cross bred cow calf revealed high rise in temperature (105.5°F), increased heart rate, labored breathing with seromucous nasal discharge, enlargement of prescapular lymph node and animal exhibited tonic clonic convulsions in response to any sudden noise. Giemsa stained thin blood smear revealed intraerythrocytic piroplasm and Koch blue bodies of *T. annulata* within the cytoplasm of lymphocytes. The species of *Theileria* was confirmed by molecular amplification of genomic DNA as *T. annulata*.

Introduction

Bovine tropical theileriosis (BTT) caused by *Theileria annulata*, an intracellular apicomplexan parasite and trans-stadially transmitted by an acarine host, *Hyalomma anatolicum anatolicum* has become a disease of paramount importance causing heavy mortality in infected animals worldwide. The disease is a serious constraint to Indian dairy industry with fatal infections in exotic cattle and substantial mortality in cross-bred and indigenous zebu cattle. Theileriosis accounts for almost 70 % bovine mortality with global losses to the tune of US $ 800 million per annum (Brown, 1997) and BTT imposes production losses of about US$ 384.3 million on Indian livestock sector (Rajendran and Ray 2014). Bovine cerebral theileriosis also termed as turning sickness is an aberrant form of infection manifested rarely by typical turning and circling movements (Van Rensburg 1976; Van Amstel 1982; Saville 2002) while symptoms of depression, head pressing, opisthotonus, nystagmus, blindness, hyperesthesia, dysmetria, dyssynergia and terminal semicomatose status are also often observed from the cases reported from different parts of the world including India (Mettam and Carmichael 1936; Flanagan and Le Roux 1957; Dabak et al. 2004; Sudan et al. 2012). *Theileria parva*, *T. taurotragi* and more rarely *T. annulata* are considered causative agents of the BCT (De Vos et al. 1981; Saville 2002; Sudan et al. 2012). In acute form of disease, animals succumb after 2–21 days but rarely in chronic case animal may survive up to 6 months (Van Amstel 1982; Lawrence et al. 2004). Previous worker in India cited the cases of *T. annulata* associated BCT based on morphology and clinical symptoms only (Alwar and Lalitha 1958; Sharma and Gautam 1973; Srivastava and Sharma 1976; Sudan et al. 2012). The present communication is the first report of molecular based confirmation of cerebral theileriosis case caused by *T. annulata* coupled with

its morphological detection, clinical manifestation, haematological alterations and therapeutic management in a cross bred cattle calf from India.

Materials And Methods

A two-month cross-bred cow calf weighing approximately 110 kg showed a high rise in temperature (105.5°F), increased heart rate 110bpm, labored breathing with seromucous nasal discharge, and enlargement of prescapular lymph node and animal exhibited tonic clonic convulsions in response to any sudden noise. Calf was showing his neck unnaturally bent backwards along with paddling movement (Fig.1). Faeces were hard and covered with bloody mucus. On enquiry from owner it was noted that the mother and her calf was brought from Jind (Haryana).

Peripheral thin blood smear was prepared from cross bred calf at the blood collection site and immediately fixed with methanol. Blood sample brought to Department of Veterinary Parasitology, College of Veterinary Science and A.H, Jabalpur and blood smear stained by the standard protocol for Giemsa staining and examined under oil immersion of microscope (Gupta and Singla 2012). Number of piroplasm infected erythrocytes was counted out of 100 erythrocytes to know the level of parasitaemia (Ramazan and Ugur 2007).

Five ml of blood was collected from the jugular vein of infected calf, and two ml was transferred to a vial containing EDTA. The remaining 3 ml blood was transferred into another vial without anticoagulant to separate serum for biochemical examination. Total erythrocyte count, haemoglobin, packed cell volume, total leucocyte count and differential count were estimated (Schalm et al. 1975). Serum creatinine, total serum proteins, serum albumin, and aspartate transaminase were estimated using a semiautomatic analyzer, as per the manufacturer's instructions and standard kits. Body hair coat was carefully examined to find out any ectoparasites and identified using standard keys (Taylor et al. 2007).

Genomic DNA extraction

Genomic DNA was extracted from blood samples as per procedure of DNA isolation kit (DNeasy blood and tissue kit, Qiagen) and stored at -20°C till further use. Genomic DNA isolated from *T. annulata* infected cattle blood showing high parasitaemia was utilized as positive control. Genomic DNA was also isolated from the whole blood of infection-free, three-day old cow calf (both microscopically and PCR negative) and used as a negative control.

PCR assay:

Molecular diagnosis of *T. annulata* was carried out by PCR as per the procedure described by d'Oliveira et al. (1995) by using the following primers: F-5'GTAACCTTTAAAAACGT3' and R-5'GTTACGAACATGGGTTT3'specifically targeting merozoite surface antigen gene.

For the PCR assay, following components were used; 12.5 µL master mix (2x), 1 µL each (10 pmol/ µL) of forward and reverse primers and 1 µL of template DNA and the volume was made up to 25µL with

nuclease-free water. The cycling conditions were: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min, and the final elongation was done at 72 °C for 10 min. The PCR product was checked for amplification by electrophoresis on a 1.5 % agarose gel and visualized using gel documentation system (Syngene, UK).

Results

Clinical observation

Clinical examination of the calf revealed pale and icteric mucous membrane of the conjunctiva. The animal was weak and emaciated. The muzzle was dry with seromucous nasal discharge from both the nostrils. The pre scapular lymph nodes were moderately swollen and enlarged. The rectal temperature was 105.5°F. Signs of respiratory distress were quite evident from labored breathing. The animal was repeatedly seen pressing head against the hard objects and exhibited tonic-clonic convulsions in response to any sudden noise and occasional circling movements (Fig. 1).

Microscopy

Microscopic examination of the of Giemsa-stained blood thin blood smears revealed Koch blue bodies (KBB) in the cytoplasm of the lymphocytes and highly pleomorphic piroplasms in the RBCs identified as *T.annulata* (Fig. 2).

PCR

Specific Primers directed amplification of PCR assay resulted in an amplicon size of 721bp in 1.2% agarose gel correspond to *T.annulata*. Nonspecific amplification was not observed in the negative control sample (Fig. 3).

Hematolobiochemical examination

The haematological values of cross bred cow calf before and after treatment of BCT were as follows: RBC ($5.0x10^6$µLVs $6.5 x10^6$), Hb (9 g/dl Vs 13.0 g/dl), WBC ($9x10^3$/ µl Vs $7x10^3$ / µl), Lymphocytes (43 % Vs 60 %); Neutrophils (53 %Vs 36 %); Eosinophils (3 % Vs 2 %) and Monocytes (1 % Vs 1 %). Biochemical parameters of naturally infected calf with BCT were as follows: aspartate transaminase (60.50 IU/L); creatinine(1.25 mg/dl) ; total protein (6.5 g/dl); albumin (2.50 g/dl); globulin (4.17 g/dl) and whereas after treatment from theileriosis were as follows: aspartate transaminase (15.50 IU/L); creatinine(1.0 mg/dl); total protein(7.80 g/dl); albumin(3.25 g/dl); globulin(4.71 g/dl).

Discussion

Giemsa stained thin blood smear revealed intraerythrocytic piroplasm and Koch blue bodies within the cytoplasm of lymphocytes confirmed as *T. annulata* (Fig. 2). PCR assay further confirmed the presence of *T. annulata* (Fig. 3). The level of parasitaemia was very high (60%) and presence of schizonts in blood

smear indicated the acute phase of disease. Further presence of schizont infected cells in blood are indicative to schizont transforming species. In case of *T. parva*, schizont stage is pathogenic causing lymphodestruction while in case of *T. annulata*, both schizont and piroplasm stages are pathogenic (Mehlhorn and Schein 1984). Otherwise, presence of only piroplasm stage in blood is indicative of benign form of theileriosis (Sivakumar et al., 2014). In the present case, occurrence of maximum number of annular form of piroplasmic stages along with presence of schizonts, which are difficult to demonstrate in chronic theileriosis and BCT, was indicative of *T. annulata* infection (Bader et al. 1986),

Species level differentiation of various *Theileria* species based on schizonts either by ultrastructure or by light microscope is quite impossible. Additionally, it is very tough for species level identification based upon piroplasm shape and size (Schein et al. 1978).

Serological methods have inherent limitations of cross reactivity (Passos et al. 1998). Nucleic acid based assays like PCR allow diagnosis of parasite at levels far below the detection limit of the frequently used parasitological techniques and help the confirmation of parasite up to species level by employing species specific primers (d'Oliveria et al. 1995; Farooq et al. 2019). Therefore, PCR assay was employed in the present case for species level identification of the *Theileria* responsible for cerebral form of theileriosis.

Primers derived from the gene encoding the 30-kDa major merozoite surface antigen of *T. annulata* were used in the present study. A 721bp fragment was amplified from the genomic DNA extracted from the cross-bred calf blood. PCR assay confirmed the identity of *T. annulata* as the etiological agent of BCT in the present study.

Based on morphological characters collected ticks from the calf were identified as *Hyalomma anatolicum anatolicum* which transmits *T. annulata* trans-stadially and it is considered as world's most damaging ticks (Giles et al. 1978). The tick is established in a majority of the middle East and South Asian countries, including India (Bhatia and Shah 2001). The most probable reason behind the BCT might be the release of toxin from tick having a suppressing effect on the reticuloendothelial system renders an animal more susceptible. It could also be due to an autoimmune disorder induced by parasites leading to intravascular agglutination of lymphoblasts (Van Rensburg 1976). More stress due to translocation may have altered the immune function and response to infection by the calf as calf along with mother was transported (Martin et al. 2011). Possible trophism of the parasite towards the central nervous system leads to lesion in endothelium responsible for lymphoblastic accumulation and venous thrombi (Bader et al. 1986). However, the severity of theileriosis depends on the inter play of parasite, vertebrate host, tick vector and environmental factors (Clift et al. 2020).

The infected calf was treated twice at the interval of 72 hrs with buparvaquone (@ 2.5 mg/kg b.wt. im) and supportive treatment for 5 days with oxytetracycline @10mg/kg b.wt im, 3 mL meloxicam with paracetamol im, 3 mL vitamin B complex and rumentas bolus 1 BD for 5 days. Blood sample was found negative after 5 days for *T. annulata* after screening with blood smear examination and PCR. The adjunction of antioxidants and antitheilerial agents may play a crucial role in salvaging the animals from lethal theileriosis.

Haematological values of Hb, PCV, and TEC were lower which may be due to various factors like severe damage caused by organisms inside the erythrocyte during their multiplication (Ganguly et al. 2015); destruction of erythrocyte infected with *Theileria* schizonts owing to immune mediated erythrophagocytosis (Uilenberg 1981); removal of piroplasm infected erythrocytes by reticuloendothelial system (Campbell and Spooner 1999); increased level of activated complement products (Omer et al. 2002) and destruction of oxidized erythrocyte by oxygen free radicals responsible for anaemia (Mbassa et al. 1994). Decreased value of serum total protein, albumin and globulin might be due to diseased lymph node resulting in extra-vascular proteinaceous fluid in body cavities (Stockham et al., 2000) in addition to liver failure (Omer et al.2003). Increased level of AST is indicative of necrosis as it is involved in amino acid and carbohydrate metabolism (Murray et al. 2000) whereas raised value of creatinine suggests muscle damage due to anemic conditions (Kaneko 1989). Though present case was of typical bovine cerebral theileriosis, however it was difficult to understand the precise mechanism of *T. annulata* induced BCT to mitigate the new threats for livestock. Nervous form is not a consistent feature of theileriosis in cattle hence, it is imperative if cattle is showing nervous symptoms along with anemia then same should be diagnosed to rule out the possibility of *T. annulata* infection. The present communication places on record the first report of molecular based confirmation of bovine cerebral theileriosis caused by *T. annulata* in a cross bred cattle calf from India.

Declarations

Funding: This work was supported by Dean, College of Veterinary Science and Animal Husbandry, Jabalpur, India.

Competing interests The authors do not have any financial or personal relationship with other people or organizations that could in appropriately influence or bias the content of the paper.

Availability of data and material Data can be shared through the corresponding author.

Code Availability Not applicable

Authors' contributions Each of the authors contributed in different aspects of the study design, data collection, data analyses and manuscript preparation. All of the authors checked and reviewed the final manuscript.

Ethical approval The study was conducted with the prior consent of the owner and sample collection was carried out in humanely manners considering animal welfare as per the standard protocol of the ethical guidelines

Consent to participate Dairy owner provided informed verbal consent and was always present during the sampling of the calf.

Consent for publication Permission has been granted by the university authorities for publication and all the authors have given their consent for publication.

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Figures

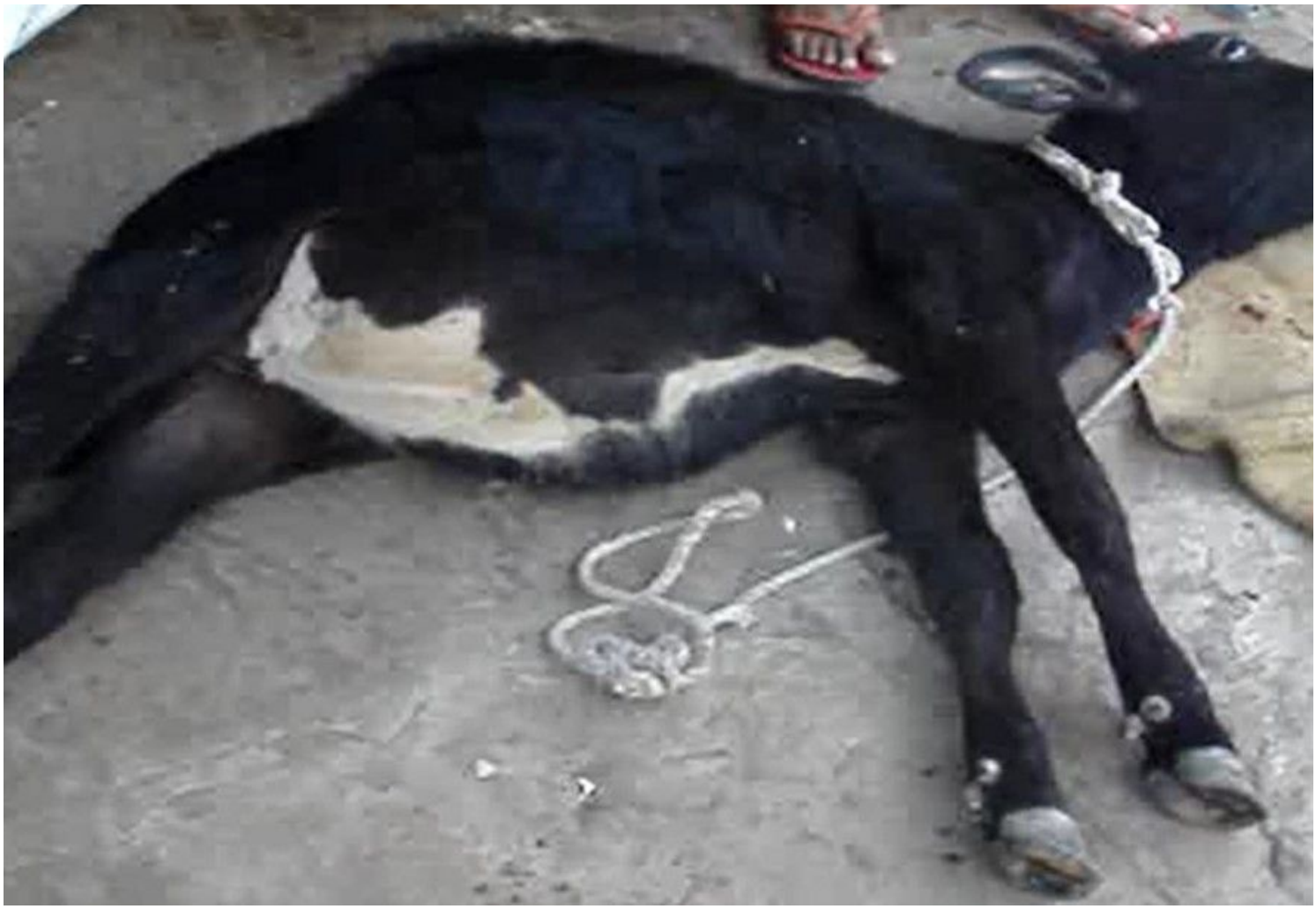

Figure 1

Cross bred cow calf showing paddling movement, opisthotonus and tonic clonic convulsions

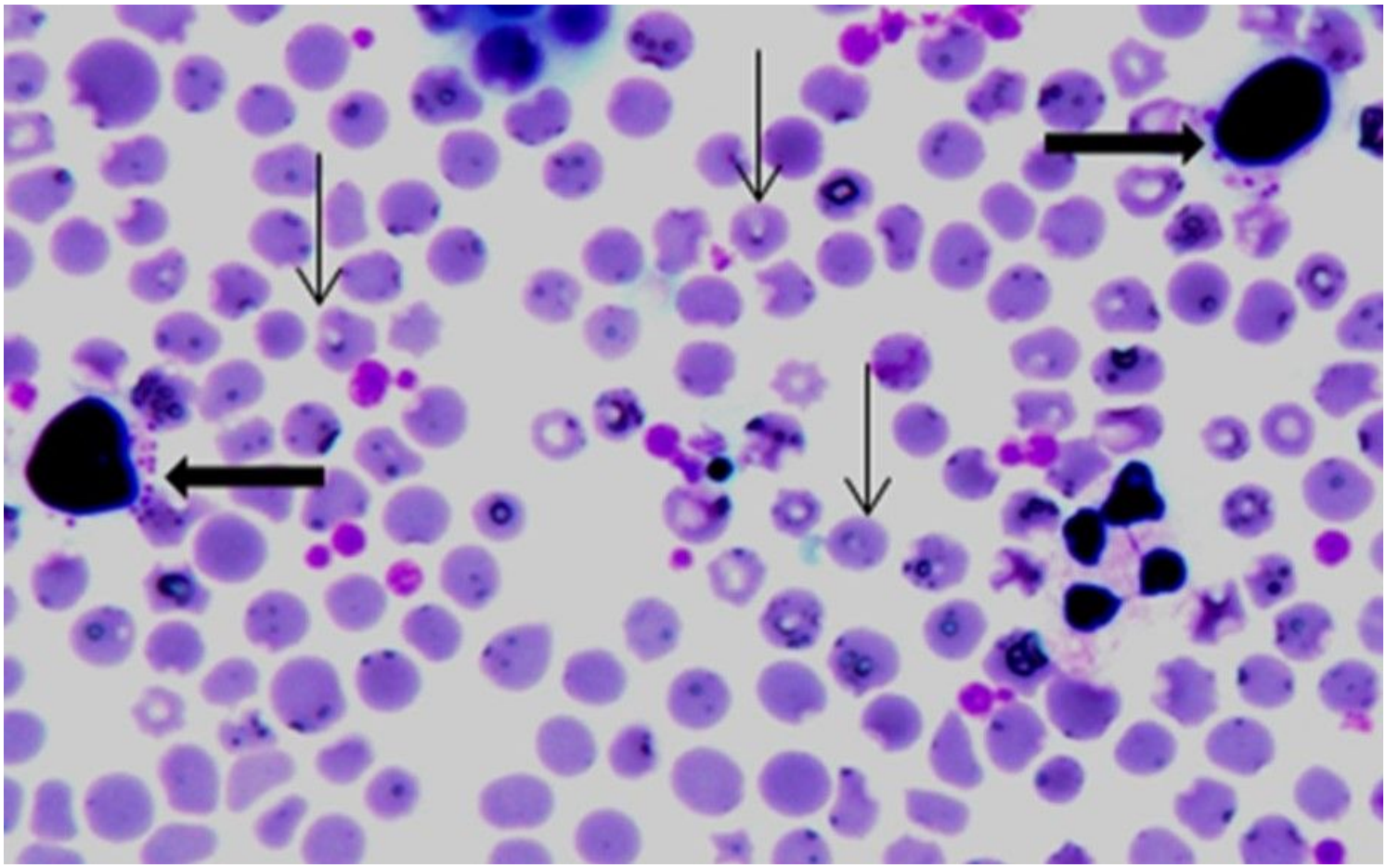

Figure 2

Koch blue body (Arrow head) in the lymphocytes and high level of T. annulata piroplasm (Arrow) in the RBC's under oil immersion lens (x1000)

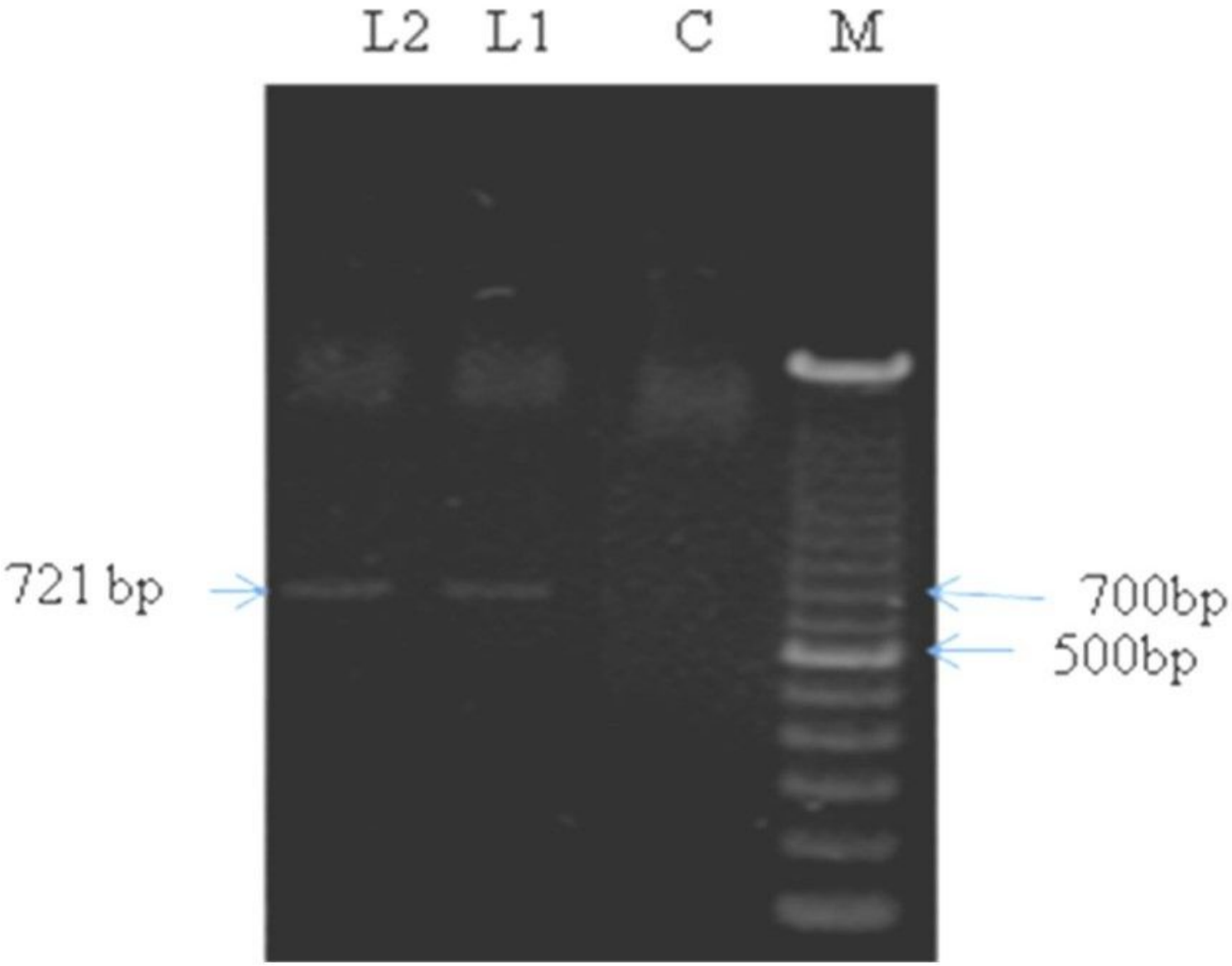

Figure 3

Agarose gel electrophoresis (1.5%) showing intact band of 721 bp fragment from genomic DNA of T. annulata Lane M : 100bp DNA ladder, Lane1 : Amplification of T. annulata genomic DNA from the blood of animal positive for infection (positive control) Lane C : Negative control (No template), Lane2 : Positive processed field samples.